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Detection of inducible nitric oxide synthase in *Schistosoma japonicum* and *S. mansoni*

X.-C. Long[1,2], M. Bahgat[1]†, K. Chlichlia[1], A. Ruppel[1] and Y.-L. Li[2]*

[1]Department of Tropical Hygiene and Public Health, University of Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany:
[2]Department of Parasitology, Tongji Medical College, Huazhong University of Science and Technology, Hangkong Lu 13, Wuhan 430030, P. R. China

Abstract

Schistosoma japonicum and *S. mansoni* were tested for reactivity with an anti-inducible nitric oxide (iNOS) antibody and the distribution of iNOS was studied by immunofluorescent tests in different stages of the parasites. Reactivity was associated with the tegument in both larval schistosomes (sporocysts and cercariae) and eggs. With adult worms, the majority of the immunofluorescence was predominantly subtegumental in *S. japonicum* and parenchymal in *S. mansoni*. Fluorescence was also observed in host tissues (snails and mouse liver). In Western blots, the enzyme of *S. japonicum* had an apparent molecular weight of about 210 kDa. The possible role of worm and host iNOS in the parasite–host interrelation remains to be clarified.

Introduction

One of the most prominent functions of nitric oxide (NO) is its participation in antimicrobial defence (Bogdan, 1997). Nitric oxide is generated from arginine by at least three isoforms of NO synthases, one of which is the inducible NO synthase (iNOS) (Xie *et al.*, 1992). Many cell types, e.g. macrophages, can express high levels of iNOS when activated by immunological stimuli, e.g. interferon gamma or tumour necrosis factor, thus leading to the killing of parasites (Liew *et al.*, 1997). Not only are protozoan parasites like *Trypanosoma* or *Leishmania* susceptible to NO-mediated killing (Green *et al.*, 1991; Norris *et al.*, 1995), but also schistosomula of *Schistosoma mansoni* can be killed by macrophages involving an arginine-dependent production of reactive nitrogen intermediates (James & Glaven, 1989), in particular of NO (Oswald *et al.*, 1994).

In addition to its role in host defence against parasites, iNOS is also involved in host pathology, although with seemingly contrasting effects. Thus, in falciparum malaria iNOS apparently contributes to cerebral and other symptoms (Clark & Cowden, 1999), and in infections with *Trichinella spiralis* iNOS is involved in the associated pathology, which is abrogated in iNOS-deficient mice (Garside *et al.*, 2000). With respect to infections with *S. mansoni*, treatment of mice with an inhibitor of iNOS exacerbated the liver pathology resulting from the granuloma formed around deposited schistosome eggs, suggesting that NO limits hepatocyte damage (Brunet *et al.*, 1999). With *S. japonicum*, however, NO was suggested to contribute at least to the early development of granulomata (Hirata *et al.*, 2001). Since granuloma formation is at the origin of most clinical symptoms associated with schistosomiasis, NO may play an important role in the schistosome–host interaction. The presence of NO synthase-like enzyme(s) in the parasite itself was also suggested by the immunostaining of adult worms of *S. mansoni* (Kohn *et al.*, 2001). In the present study, the presence of iNOS in *S. japonicum* is tested for the first time and compared with *S. mansoni*, including adult worms and larval stages developing in the intermediate snail host and also in the eggs.

Materials and methods

Immunofluorescent tests with snail and liver sections

Snail sections were prepared and tested essentially as described by Bahgat *et al.* (2001). Paraffin sections were

*Author for correspondence
Fax: 86 27 83692608
E-mail: lylong@mails.tjmu.edu.cn
†Present address: Department of Medicinal Chemistry, National Research Centre, Dokki, Cairo, Egypt

prepared from schistosome-infected snails: *Oncomelania hupensis* (intermediate host of *S. japonicum*) were collected in an endemic area of the Hubei province, China and *Biomphalaria glabrata* (intermediate host for *S. mansoni*) was a laboratory strain maintained in Heidelberg, Germany. Snails shedding cercariae were used to prepare the soft tissues, and all residual pieces of shells were carefully removed. Tissues were fixed in 4% formaldehyde for 48 h and embedded in paraffin. Sections (6 μm) were cut, rinsed in water, placed on a drop of xylol on a glass slide, left to dry for 30 min at 37°C and stored at room temperature until use. Before performing immunofluorescence tests, sections were de-paraffinized by rinsing with decreasing concentrations of ethanol and incubated in phosphate (0.01 M) saline (PBS; pH 7.2). Sections were covered with a rabbit antibody directed against mouse iNOS (Sigma, Deisenhofen, Germany) diluted 1:400 in PBS and incubated in a humid chamber for 1 h at 37°C. Normal rabbit serum was used for control tests. The slides were rinsed, washed three times for 10 min with PBS and dried at room temperature, followed by incubation for 1 h with FITC-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories; München, Germany) diluted 1:100 in PBS containing 2% Evan's blue (Sigma). The slides were rinsed, washed as before, covered under glycerin (Merck, Darmstadt, Germany; 20% in PBS) with a cover slide, and viewed in a fluorescence microscope (Axioscop; Zeiss, Göttingen, Germany). Green and green-yellow fluorescence were considered positive reactions. A red background was considered negative.

Livers of *S. japonicum*-infected mice were cut into pieces of a few millimetres in diameter, fixed in formaldehyde, and further processed for immunofluorescence as described above for the snail tissues.

Immunofluorescent tests with worm sections

Cryostat sections of adult worms were prepared and processed for immunofluorescence as described by Ruppel *et al.* (1985): *S. japonicum* (a strain from Hubei, China) and *S. mansoni* (a laboratory strain from Puerto Rico maintained in Heidelberg) were collected by portal perfusion from infected NMRI mice, at least 40 days after infection (Ruppel *et al.*, 1990). Freshly collected worms were kept overnight at 4°C in Dulbecco's modified Eagle Medium supplemented with 5% heat-inactivated newborn calf serum (Gibco-Invitrogen, Karlsruhe, Germany). This treatment induced worms to vomit residual mouse blood from their gut, which otherwise would result in non-specific fluorescent reactions when testing the sections with anti-mouse immunoglobulins. After incubation, worms were washed once in medium, then embedded in tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and frozen. Cryostat sections (6 μm) were prepared, placed on slides, air-dried overnight at room temperature and stored at −20°C until use. Immunofluorescence tests with anti-iNOS antibody were performed as described above for the snail sections.

All tests were performed with three independent replicates.

Western blots

Adult *S. japonicum* worms which had been induced to vomit mouse blood from their gut, were washed in medium without serum, homogenized in ice-cold NaCl (0.86%) containing 1–2 μg ml^{-1} of aprotinin, 1–2 μg ml^{-1} of leupeptin, 100 μg ml^{-1} of PMSF and 0.1 mM of DTT, and centrifuged (10,000 *g*, 4°C, 15 min). The supernatant was retained and the protein concentration determined (BCA kit; Pierce, Rockford, USA). Samples with 30 μg of protein were electrophoresed under reducing conditions on 7% SDS-polyacrylamide gels as previously described by Ruppel *et al.* (1985). Proteins were electrophoretically transferred to nitrocellulose sheets and these were blocked (overnight, 4°C) with defatted milk (5% powder in PBS containing 0.3% Tween20; PBST). Immunoreactions were performed with a rabbit anti-iNOS antibody (Sigma; diluted 1:3000 in PBST), followed after washing by horseradish-conjugated anti-rabbit IgG (Bio-Rad; 1:2000 in PBST) and detected, again after washing, with the substrate diaminobenzidine. Pre-stained proteins (Invitrogen, Karlsruhe, Germany) were used as molecular weight standards.

Results

Sporocysts and cercariae of *S. japonicum* and *S. mansoni* in sections of the respective intermediate snail hosts were tested with an antiserum against iNOS (anti-iNOS). A strong fluorescence was observed in association with the surface of the larvae of each species (for *S. mansoni*, see fig. 1A; *S. japonicum* not illustrated). Some fluorescence was also observed in the tissues of *B. glabrata*, and to a lesser degree, in *O. hupensis*, irrespective of whether these snails were infected with schistosomes or not infected (not illustrated). Control tests with normal rabbit serum showed no detectable fluorescence.

Adult worm sections of both *S. japonicum* (fig. 1B) and *S. mansoni* (fig. 1C) showed fluorescence when tested with anti-iNOS, but not with normal rabbit serum. The localization of the iNOS-specific staining was, however, different between both species. For *S. japonicum*, fluorescence was observed predominantly in a subtegumental position, but much less in the parenchyma and occasionally in the gut. In contrast, the staining of *S. mansoni* worms was mainly located deeper in the parenchyma, but less in a subtegumental position.

Liver sections of mice infected with *S. japonicum* were also tested with anti-iNOS. Fluorescence was associated with the parasite eggs and also with the surrounding liver granulomata and blood vessels (fig. 1D), whereas normal rabbit serum showed no reaction (not illustrated).

Western blot analysis of adult *S. japonicum* proteins using an antiserum specific for mouse iNOS revealed a double band with the dominant reaction at 210 kDa and a minor one at about 200 kDa (fig. 2).

Discussion

The present work has extended immunoreactivity studies on iNOS in adult *S. mansoni* (Kohn *et al.*, 2001) to the larval stages of *S. mansoni*, and for the first time to the developmental stages of *S. japonicum*. The reactivity of antibodies against mouse iNOS was observed with

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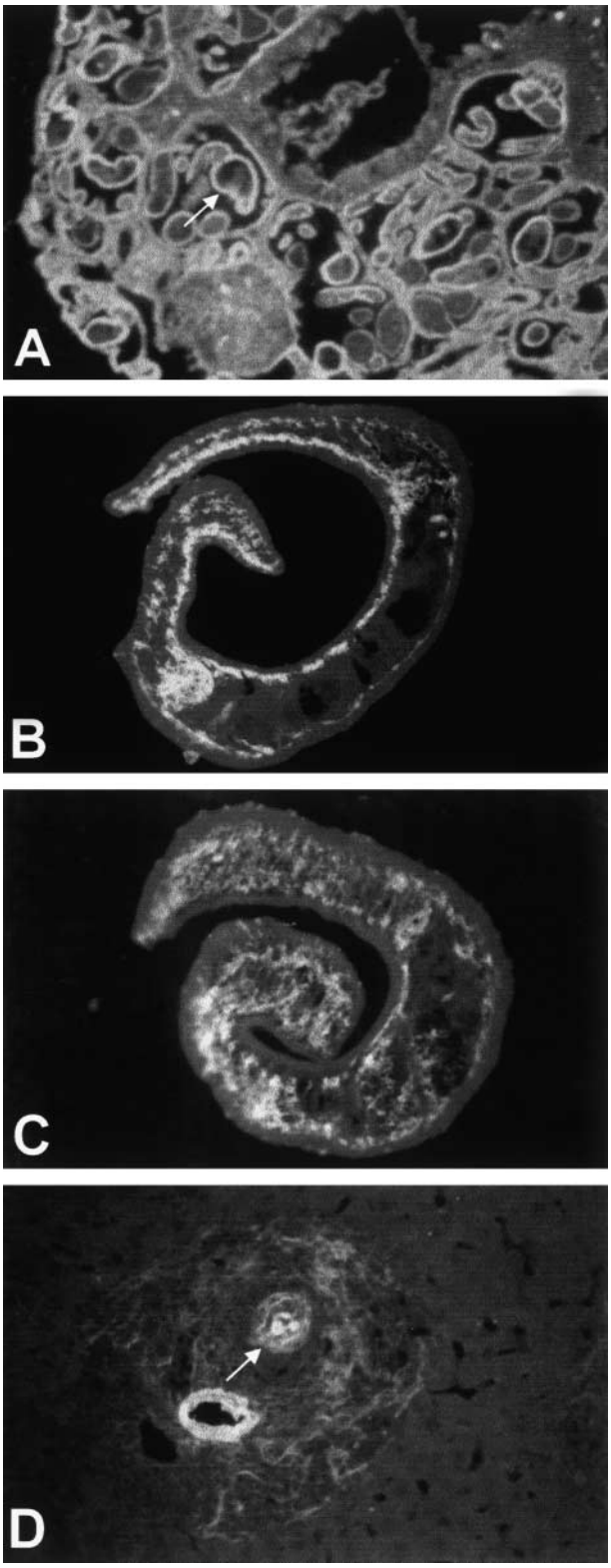

Fig. 1. Immunofluorescence studies with sections of various developmental stages of schistosomes using an antibody directed against mouse inducible nitric oxide synthase. A. Paraffin section of *Biomphalaria glabrata* infected with *Schistosoma mansoni*; the arrow points to the head of a cercaria showing florescence on the surface including its tail. B. Cryostat section of an adult male *Schistosoma japonicum*. C. Cryostat section of an adult male *S. mansoni*. D. Paraffin section of *S. mansoni*-infected mouse liver; the arrow points to the schistosome egg, which is surrounded by the granulomatous host reaction; the strong fluorescence in the lower left is associated with a blood vessel.

cryostat sections of adult male worms from both species, although with somewhat different distributions. Fluorescence was restricted predominantly to a subtegumetal location in *S. japonicum*, but to the parenchyma in *S. mansoni*. In contrast to Kohn *et al.* (2001) no fluorescence was associated with the adult male dorsal surface of *S. mansoni*, and in particular with the tubercles. Similarly, the surface of *S. japonicum* was devoid of fluorescence, but it should be recalled that male worms of the latter species lack tubercles (McLaren, 1980). The occasional localization of anti-iNOS reactivity in the gut apparently agrees with Kohn *et al.* (2001). However, the possible contamination by ingested mouse cells cannot be ruled out, although vomiting of the worms was induced prior to microscopic preparation. The anti-iNOS reactivity observed here for *S. japonicum* in Western blots at about 210 kD is comparable with the molecular weight of about 200 kD reported by Kohn *et al.* (2001) for iNOS of *S. mansoni*. Taken together, these results indicate that adult schistosomes possess an iNOS-like enzyme in an internal localization.

Sporocysts and cercariae in sections of snails infected with either schistosome species showed an anti-iNOS specific fluorescence associated with the larval tegument. Whether the NO, which may potentially be generated at the larval surface, acts on the snail host, remains unknown. Conversely, iNOS-specific fluorescence was also evident in the tissues of infected as well as uninfected snails (not illustrated). Although the microscopical resolution in the present study was not sufficient to associate the reactivity with particular cells, it is relevant to note that NO generated by haemocytes from schistosome-resistant snails (*B. glabrata*) contributes to the *in vitro* killing of sporocysts (Hahn *et al.*, 2001). The simultaneous demonstration of iNOS-specific fluorescence in the parasite and in its snail host suggests that both organisms also possess the ability to deal with toxic nitrogen species. The interaction between the developing parasite and the host's defence still appears to be unclear.

Similarly, anti-iNOS reactivity was observed in host cells of the granulomata surrounding the eggs of *S. japonicum* as well as in the eggs themselves. The reported effects of NO or of iNOS on hepatic damage in murine schistosomiasis mansoni were not consistent. Mice treated with an inhibitor (aminoguanidine) of iNOS 'exhibited exacerbated liver pathology' (Brunet *et al.*, 1999), while treatment with another iNOS inhibitor (L-NAME) 'led to a marked decrease of hepatic injury' (Abdallahi *et al.*, 2001). The present results show that host cells express iNOS around the eggs of *S. japonicum* and agree with those of Hirata *et al.* (2001) who found iNOS expressed around the eggs of this parasite. However we cannot deduce a role for iNOS from the host cells on pathology. The origin of iNOS from the parasite egg

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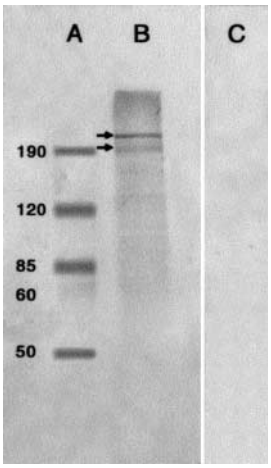

Fig. 2. Western blot of adult *Schistosoma japonicum* proteins. Lane A, molecular weight markers; lane B, schistosome proteins tested with a rabbit antibody directed against mouse inducible nitric oxide synthase; lane C, schistosome proteins tested with normal rabbit serum. The arrows indicate reactivity of the antibody, which dominates with a band of about 210 kD.

further complicates the understanding of the role of NO or iNOS in schistosomiasis.

In conclusion, the evidence of iNOS expression by *S. japonicum* in larval stages and adult worms as well as their eggs may indicate that iNOS of the parasite is also targeted towards the outside of the parasite and interacts with its host.

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